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(54) Title: ELECTROPHILIC POLYETHYLENE OXIDES FOR THE MODIFICATION OF POLYSACCHARIDES, POLYPEPTIDES (PROTEINS) AND SURFACES			
(57) Abstract			
Poly(ethylene glycol) mixed carbonates are synthesized by conversion of polyethylene glycol first to the chloroformate then by reaction with the hydroxyl group of N-hydroxybenzotriazole or 2-hydroxypyrimidine or N-hydroxy-2-pyrrolidone. The structural formulae for these mixed carbonates allows for them to smoothly react with the amino groups in aminoglycans and protein and amino containing surface to form stable, hydrolysis resistant carbamate linkages.			<p>Chemical structures of various poly(ethylene glycol) mixed carbonates labeled 1 through 8:</p> <ul style="list-style-type: none"> 1: A repeating unit of a poly(ethylene glycol) carbonate with a 2-hydroxypyrimidine (HMPA) side chain. 2: A repeating unit of a poly(ethylene glycol) carbonate with a 2-hydroxypyrimidine (HMPA) side chain. 3: A repeating unit of a poly(ethylene glycol) carbonate with a 2-hydroxypyrimidine (HMPA) side chain. 4: A repeating unit of a poly(ethylene glycol) carbonate with a 2-hydroxypyrimidine (HMPA) side chain. 5: A repeating unit of a poly(ethylene glycol) carbonate with a 2-hydroxypyrimidine (HMPA) side chain. 6: A repeating unit of a poly(ethylene glycol) carbonate with a 2-hydroxypyrimidine (HMPA) side chain. 7: A repeating unit of a poly(ethylene glycol) carbonate with a 2-hydroxypyrimidine (HMPA) side chain. 8: A repeating unit of a poly(ethylene glycol) carbonate with a 2-hydroxypyrimidine (HMPA) side chain.

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DESCRIPTION

Electrophilic Polyethylene Oxides for the
Modification of Polysaccharides, Polypeptides
(Proteins) and Surfaces

Field of the Invention

The present invention relates to compositions for coating substrate surfaces which have polyoxyalkylene tethers attached thereto. These tethers allow bioactive 5 compounds such as polysaccharides, polypeptides, proteins and other pharmaceuticals to be covalently attached to the surface. One such bioactive compound is heparin which renders such surfaces thromboresistant. Membrane coatings including these thromboresistant surfaces are particularly 10 suited for use in conjunction with biomedical devices.

Background of the Invention

Publications and other references referred to herein are incorporated herein by reference and are numerically referenced in the following text and respectively grouped 15 in the appended Bibliography which immediately precedes the claims.

The present invention relates to the chemical modification of aminoglycans, proteins and amine coated surfaces by means of the covalent bonding of polymer chains of 20 polyoxyalkylenes, such as polyethylene oxide (also called polyethyleneglycol) and polypropylene glycol.

Polyethylene glycol (PEG) use in biotechnology and biomedical applications continues to expand and has recently been reviewed (1). Modification of enzymes (2), 25 RGD peptides (3), liposomes (4), and CD4-IgG glycoprotein (5) are some of the recent advances in the use of polyethylene glycol. The modification of toxicity, pharmacokinetics, biodistribution and other biofunctions are a number of the promising areas for the use of this simple 30 polymer. Surfaces treated with PEG have been shown to

resist protein deposition and have improved resistance to thrombogenicity when coated on blood contacting biomaterials (6). Accordingly, application of PEG based coatings to various polymeric materials especially with 5 respect to "continuous" coating of microporous hollow fiber or other plastic parts would be very useful for medical devices.

Electrophilic activated polyoxyalkylenes such as PEG for continuous coating applications should satisfy the 10 following requirements:

1. The rate of reaction between an amino group coated surface and/or an amino containing biomolecule with an electrophilically activated PEG should have an fast reaction rate under mild conditions.
- 15 2. Ideally, the electrophilically activated PEG should have an appropriate hydrolysis half-life in water at pH values of 7.5-8.5. This is especially important with respect to polymeric substrates to be coated that can not withstand exposure to organic solvents.
- 20 3. Formation of a covalent bond between an amino-containing biomolecule and the electrophilically activated PEG should be demonstrated by spectroscopic means such as nuclear magnetic resonance an/or infrared spectroscopy to demonstrate that the chemistry proceeds as expected.
- 25 4. Should organic solvents be used in the coating process, stability of the electrophilically activated PEG should be appreciable for economical reasons.
- 30 5. Reaction of the electrophilically activated PEG with the biological molecules of interest should be site directed so that crucial receptor and/or active sites are not blocked. In turn, retained biological function should be demonstrated by an appropriate assay.
- 35 6. Quality or functionality of the electrophilically activated PEG should be easily determined by rapid spectroscopic means during a manufacturing process.
7. The leaving group released upon acylation of amino groups should have high solubility in the reaction

medium, minimal adsorption to the modified substrate and be non-toxic ideally.

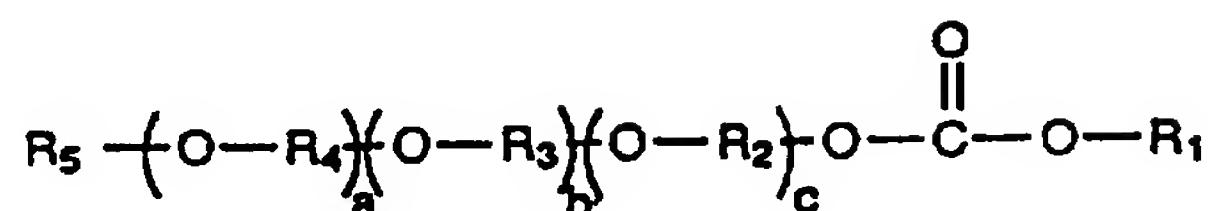
8. The electrophilically activated PEG should have long term stability for shelf life storage purposes.

5 9. The covalent bond formed should be hydrolysis resistant under both the coating conditions and subsequent "actual use" conditions.

In order to covalently bond, the hydroxyl group of PEG must be "activated". This has been reported as 10 accomplished by the use of a number of reactive functional groups including cyanurylate (7-9), tresylate (10-11), N-hydroxysuccinimide derived active esters (12-17), carbonates (18-20), imidazolyl formates (21-22), 4-dithiopyridines (23), isocyanates (24) and epoxides 15 (25). Each of the above functional groups possess disadvantages which range from leaving group toxicity, conjugates that are prone to hydrolysis under physiological conditions and slow reaction rate in the conjugation process. Radiolabeled urethane-PEG derivative 20 stability has been demonstrated under a variety of physiological conditions (26). The hydrolysis resistant urethane bond produced by sufficiently reactive PEG carbonates may offer considerable advantage in avoiding hydrolysis of the conjugation covalent bond. To date, 25 literature reports on the use of PEG carbonates have focused on the modification of protein or polypeptides. Driven by our interest in developing a continuous coating process for covalently bound heparin on a microporous hollow fiber surface, we explored the reactivity of 30 various PEG carbonates with the aminoglycan D-glucosamine and several commercially available sodium heparins.

Summary of the Invention

The present invention provides for compounds having the formula:



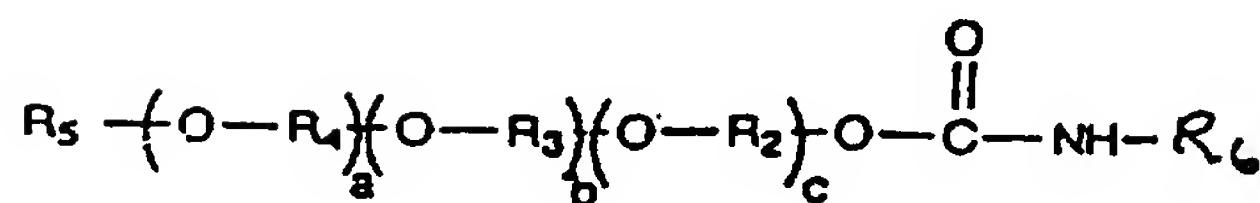
(I)

5 wherein R_1 is selected from the group consisting of an N-benzotriazole group, an N-2-pyrrolidinone group, and an 2-oxypyrimidine group; R_2 , R_3 and R_4 are independently selected lower alkylene groups, preferably of about 2 to about 3 carbon atoms, and may be the same or different; R_5
10 is selected from the group consisting of hydrogen, methyl, a carbonyloxy-N-benzotriazole group, a carbonyloxy-N-2-pyrrolidinone group, and a carbonyl-2-oxypyrimidine; a is an integer from 1 to 1000 and each of b and c is an integer from 0 to 1000, and a , b and c are
15 selected so that the sum of a , b , and c is an integer between 3 and 1000.

Preferred are compounds of formula (I) wherein each of R_2 , R_3 and R_4 is independently $-CH_2CH_2-$ or $-CH_2CH(CH_3)-$ or a combination thereof.

20 These compounds may be homobifunctional or heterobifunctional and are suitable for modifying a variety of bioactive compounds as well as acting as a tether to link a bioactive compound to a membrane or polymeric surface.

25 According to a preferred aspect, the present invention further provides for the production of a modified bioactive compound such as a polysaccharide or polypeptide or other pharmaceutical that has conjugated to it via an urethane bond at least one molecule of polyoxyethylene
30 having the structure:



(II)

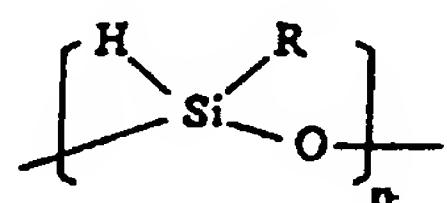
where R_5 is selected from the group consisting of hydrogen, methyl, a carbonyloxy-N-benzotriazole group, a carbonyloxy-N-2-pyrrolidinone group, or a carbonyl-2-oxypyrimidine group, R_2 , R_3 and R_4 are ethylene, R_6 is a bioactive compound selected from an aminoglycan polysaccharide, a polypeptide, a protein or other reactive pharmaceutical compound, and a , b , and c are as defined in connection with formula (I). These modified bioactive compounds having covalently attached polyoxalkylene groups have a variety of uses in vivo, since such substances may exhibit a reduced rate of kidney clearance and/or enhanced association with cells, and in certain instances decreased immunogenicity.

In addition, according to an additional aspect, this invention provides for a method for the covalent bonding of aminoglycan polysaccharides, polypeptides or proteins using a polyoxalkylene, such as PEG, activated with a carbonyloxy-N-benzotriazole group, a carbonyloxy-N-2-pyrrolidinone group, or a carbonyl-2-oxypyrimidine group to any surface in possession of reactive amino groups or other suitable nucleophiles. In addition, the heterobifunctional polyoxalkylenes produced thereby possess different activating groups at each end of the polyoxalkylene chain are useful as linkers or tethers. Use of these tethers allow anchoring of the aminoglycan polysaccharides, polypeptides or proteins to other substances having an amino or appropriate nucleophile group.

Thus, the present invention includes a method for the covalent bonding of a bioactive compound selected from an

aminoglycan polysaccharide, a peptide, a protein or other pharmaceutically active substance to a polymeric surface using a polyoxyalkylene tether which comprises contacting a substrate having an amine-grafted polymeric surface 5 having free amino groups with a compound of formula (I) to give a modified polymeric surface having activated polyoxyalkylene groups covalently bonded thereto; and contacting the modified polymeric surface with the bioactive compound.

10 According to an especially preferred aspect, the present invention provides a coating which comprises a membrane formed from the plasma polymerization of hydrocyclosiloxane monomer of the general formula:



where R is an aliphatic group having 1 to about 5 carbon 15 atoms and n is an integer from 2 to about 10, covalently linked by a carbamate linkage to one end of a polyoxyalkylene tether wherein the tether is covalently linked at its other end by a carbamate linkage to a bioactive molecule. Where the bioactive molecule is 20 selected from compounds having antithrombotic or thrombolytic properties such as heparin, tissue plasminogen activator, streptokinase, prostaglandins and antiplatelet drugs, coatings having enhanced thromboresistance are provided. According to an alternate preferred aspect, the 25 bioactive compound is a metal chelator such as deferoxamine.

Coatings prepared from plasma polymerization of a hydrocyclosiloxane monomer selected from the group consisting of 1,3,5,7-tetramethylhydrocyclotetrasiloxane, 30 1,3,5,7,9-pentamethylhydrocyclopentasiloxane, 1,3,5,7,9,11-hexamethylhydrocyclohexasiloxane, and a mixture of 1,3,5,7,9-pentamethylcyclopentasiloxane and

1,3,5,6,9,11-hexamethylcyclohexasiloxane monomers are especially preferred.

Brief Description of the Drawings

Figure 1 depicts certain electrophilic PEG analogs according to the present invention.

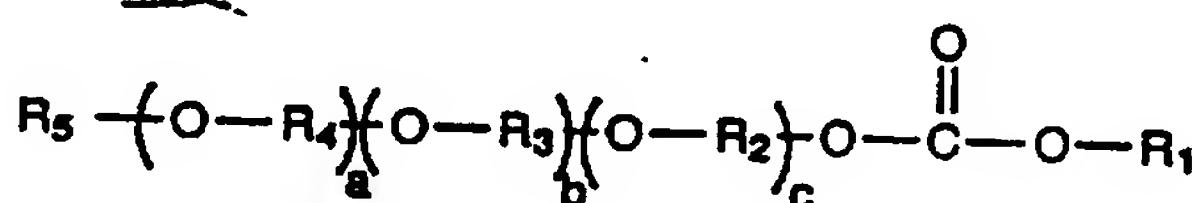
Figure 2 depicts a reaction scheme of a PEG analog of the present invention with D-glucosamines and N-trimethylsilylallylamine.

Figure 3 depicts natural abundance (inset) and 95% ^{13}C enriched PEG 1 - heparin conjugate Carbon NMR spectrum.

Figure 4 depicts gel filtration high pressure liquid chromatograms of sodium heparin and PEG conjugates derived from 1, 2, 3, 4 and 5.

Detailed Description of the Invention

The present invention provides several carbonate based polyalkylene oxides 1, 2, and 3 (see Figure 1) having the general formula:



(I)

wherein R_1 is an N-benzotriazole group, an N-2-pyrrolidinone group, or an 2-oxypyrimidine group; R_2 , R_3 and R_4 are independently selected lower alkylene groups, and may be the same or different; R_5 is selected from hydrogen, methyl, a carboxyloxy-N-benzotriazole group, a carboxyloxy-N-2-pyrrolidinone group, or a carbonyl-2-oxy-pyrimidine group; a is an integer between 1 and 1000; and each of b and c is an integer between 0 and 1000, such that the sum of a , b , and c is between 3 and 1000. Suitable lower alkylene groups include those having about 2 to about 3 carbon atoms. Preferred are compounds of formula (I) where R_2 , R_3 and R_4 is $-(\text{CH}_2\text{CH}_2)-$ or $-\text{CH}_2\text{CH}(\text{CH}_3)-$ or any combination thereof. More preferably R_2 , R_3 and R_4

are ethylene. According to a preferred aspect a, b, and c are selected so as to give a molecular weight for the PEG moiety of about 500 to about 20,000, more prpeferably from 3000 to 4000.

5 Preferred Polyoxyalkylene Analogs

According to one aspect, this invention is directed to the synthesis and use of the new carbonate PEG analogs, which are depicted in Figure 1 as 1, 2 and 3 and include polyoxyethylene bis-(2-hydroxypyrimidyl) carbonate 3, 10 polyoxyethylene bis-(N-hydroxybenzotriazoyl) carbonate 1, polyoxyethylene bis-(N-hydroxy-2-pyrrolidinonyl) carbonate 2 for the modification and surface binding of aminoglycan polysaccharide or protein. According to a preferred aspect, chain length of the PEG portion is selected so as 15 to correspond with a molecular weight of about 500 to about 20,000, more preferably from about 3000 to about 4000.

Amino group covalent bonding of the carbonates 1, 2, 3, the PEG chloroformate 4 and the N-hydroxysuccinimide 20 derived PEG carbonate 5 (See U.S. Patent 5,122,614 to Zalipsky) may be conveniently demonstrated using ¹³C nuclear magnetic resonance spectroscopy coupled with the synthesis of ¹³C enriched carbonate PEG analogs. Dissolution of a protein such as human serum albumin or 25 the aminoglycans such as D-glucosamine, D-glucosamine-6-sulfate or sodium heparin, in water at pH 8.5 followed by addition of solid carbonate PEG at ambient room temperature for 30 minutes followed by ultrafiltration (Human serum albumin-PEG conjugate and 30 sodium heparin used a 10,000 mw cut-off membrane and a 500 mw cut-off membrane for D-glucosamine and D-glucosamine-6-sulfate) afforded after lyophilization white solids. Reaction was also conducted using N-trimethylsilylallylamine in ethanol (See Figure 2). 35 These solids were characterized using ¹H and ¹³C nuclear

magnetic resonance for signs of urethane bonding (See Figure 3 and Tables 1 and 4).

The approximate half-life of each of the PEG carbonates at pH 7.5-8.5 was determined using FT-infrared 5 spectroscopy (See Table 2). Hydrolysis could not be determined for poly(oxyethylene) bis-(2-hydroxypyrimidyl) carbonate 3 since the N-migration product 6 predominated in the absence of an amino nucleophile. Analog 2 demonstrated extended half-life in water when compared to 10 1 or 5. This extended lifetime in water will prove useful in coating plastic parts that can only withstand aqueous coating conditions. Alcoholic solvents such as methanol and ethanol rapidly react with 1 forming the alkylcarbonate. However, excellent stability of 1 has 15 been demonstrated for up to 6 months in dichloromethane unprotected from atmospheric moisture. Carbonates 2 and 3 as well as 5 have excellent stability in ethanol as monitored by ¹H NMR spectroscopy.

Since the aminoglycan such as heparin and other 20 proteins and peptides are substrates for modification, heparin amino content was determined using the fluoroprobe fluorescamine. Various commercially available and chemically modified heparins were analyzed for primary amino content (See Table 3). All heparins examined 25 revealed sufficient amino groups available for chemical modification as demonstrated by synthesis of 95% ¹³C enriched carbonates and reaction with various heparins (Table 4).

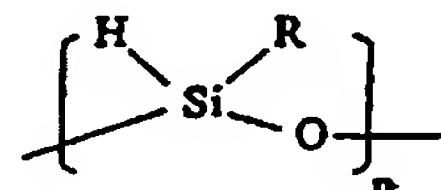
Size exclusion HPLC chromatography was utilized in the 30 case of sodium heparin to determine the degree of incorporation of PEG into the polysaccharide chain. (See Table 4 and Figure 4). Based on retention times when compared to the starting heparin, incorporation of PEG into the heparin was small in line with the number of 35 amino groups present. Formation of carbonate groups between PEG and heparin hydroxyl groups however, can not be ruled out based on the ¹³C NMR data.

Inhibition of Factor X activity in the presence of Antithrombin III ("AD") was demonstrated for the PEG-urethane-heparin conjugates. The ability of the conjugates to inhibit Factor X in the presence of 5 Antithrombin III is decreased by PEG conjugation. Sufficient activity remains, indicating that the AT III binding site has not totally disrupted.

A tetramethylcyclotetrahydrosiloxane plasma deposited, N-trimethylsilylallylamine plasma grafted microporous 10 hollow fiber was treated with these PEG carbonates and evaluated. Fiber was coated with polyoxyethylene bis-(N-hydroxybenzotriazolyl) carbonate 1 (solvent: dichloromethane, dwell time 10 minutes), a dichloromethane wash, followed by treatment with a pH 8.5 solution of 15 sodium Dowex cation exchange treated sodium heparin (dwell time 10 minutes) and a water wash. Ability to inhibit Factor X in the presence of AT III was evaluated for the fiber surface using the chromogenic substrate S-2222. Surface activity relative to the USP K2 heparin standard 20 ranged from 7.8-14.0 milliInternational units per cm² (Table 5).

Preferred Polyoxyalkylene Modified Membranes and Coatings

According to a preferred aspect, polyoxyalkylene modified polymeric membranes or coatings are provided that 25 comprise a membrane or coating on a substrate formed from the plasma polymeration of a hydrocyclosiloxane monomer of the general formula



where R is an aliphatic group having 1 to about 5 carbon atoms and n is an integer from 2 to about 10, covalently 30 linked by a carbamate linkage to one end of a polyoxyalkylene tether wherein the tether is covalently

linked at its other end by a carbamate linkage to a bio-active molecule.

Commonly assigned United States Patent application Serial No. 08/152,176, "Hydrocyclosiloxane Membrane 5 · Prepared by Plasma Polymerization Process", filed November 12, 1993, the disclosure of which is incorporated by reference herein, describes preferred membranes and their preparation. The commonly assigned and concurrently filed United States Patent application "Plasma Grafting of 10 Surfaces Using N-protected Amines" describes preferred N-protected amines and methods for their use in plasma grafting to give amine grafted membranes. These amine grafted membranes may then be conveniently reacted with the electropolytic carbonate-polyoxyalkylenes described 15 herein to give polyoxyalkylene modified membranes or coatings. In turn, these may be reacted with appropriate bioactive compounds to give the polyoxyalkylene modified membranes or coatings having a polyoxyalkylene tether linking the bioactive compound to the membrand or coating.

20 According to an expecially preferred aspect, the bioactive molecule is heparin and the resulting membrane or coating has demonstrated improved thromboresistance. Such membranes or coatings are suitable for use in biomedical devices, including intravascular oxygenators.

25 To assist in understanding the present invention, the following examples are included which describe the results of a series of experiments. The following examples relating to this invention should not, of course, be construed as specifically limiting the invention and such 30 variations of the invention, now known or later developed, which would be within the purview of one skilled in the art are considered to fall within the scope of the present invention as hereinafter claimed.

Example 1: Synthesis of Poly(oxyethylene)bis-(N-hydroxybenzotriazolyl) Carbonate 1 - A General Synthesis Procedure for PEG Carbonates

A toluene azeotroped solution of polyethylene glycol MW 3350 (1000 grams, 0.299 mol) in 2 liters of warm toluene under nitrogen was transferred by canula slowly over 1 hour to a 500 mL solution of phosgene (Caution: Use proper ventilation) in toluene (0.965 mol) and solid sodium carbonate (63.6 g, 0.600 mol) at 0°C. The reaction mixture was stirred for 2 hours under a nitrogen environment. The ice bath was drained, a water aspirator was attached and vacuum applied with vigorous stirring. The bath was filled with warm water and the vacuum continued for 30 minutes. The solution was diluted with 2 liters of anhydrous dichloromethane to aid in filtration and quickly filtered through a coarse porosity sintered glass funnel. The solution of crude chloroformate was concentrated by rotary evaporation with a water bath temperature of 40-42°C.

The resulting semisolid was immediately dissolved in 2.5 liters of dry acetonitrile and transferred to a 5 liter 3-neck round bottom flask under a nitrogen environment. This flask was cooled down to 0-3°C with vigorous stirring as measured by an internal thermometer. The N-hydroxy compound, N-hydroxybenzotriazole monohydrate (130.4 g, 0.965 mol) was dissolved into 100 mL dry acetonitrile and triethylamine (135 mL, 0.965 mol). This solution was added dropwise to the cooled solution of chloroformate at such a rate that the internal temperature did not exceed 5°C. The reaction was stirred for 15 minutes, then the mixture was filtered through a coarse porosity sintered glass funnel to remove triethylamine hydrochloride. The reaction mixture was concentrated by rotary evaporation to remove the acetonitrile with the water bath not exceeding 40 °C.

The thick oil/suspension was then dissolved in 2 liters of dichloromethane and 2 liters of distilled

deionized water. The mixture was poured into a separatory funnel and the aqueous phase extracted with three 1 liter portions of dichloromethane. The pooled organic phases were washed successively with 2.5% aqueous HCl, 1 M aqueous sodium bicarbonate and water. The organic phases were dried over anhydrous magnesium sulfate and filtered by suction through a coarse porosity sintered glass funnel. The dichloromethane was removed by rotary evaporation affording the crude carbonate as an oil. This oil was poured into 2 mechanically stirred, 5 liter Erlenmeyer flasks (approximately on half of the oil in each flask) each containing 4 liters of ice cold ethyl acetate. Dichloromethane (100 mL) was used to rinse the flask of carbonate which was poured into the ethyl acetate. Precipitation of the carbonate was aided by placing the flasks in an explosion proof freezer overnight. The solid carbonate was then collected by suction filtration, washed with a minimum of ice cold ethyl acetate and dried in vacuo (916 g). The filtrate can be reduced in vacuo and precipitated in ethyl acetate to afford a second crop of carbonate. The yield was 94% of a granular off-white solid.

Spectral data: IR (TF, NaCl) 2942, 2885, 1773 and 1754 (carbonate carbonyls), 1494, 1465, 1434, 1359, 1344, 1302, 1281, 1258, 1241, 1148, 1113, 1061, 962, 843, 767, 748 cm^{-1} ; ^1H NMR (CDCl_3) δ 8.22 (ddd, $J = 1, 2$ and 8.4 Hz, 1 H, aromatic H), 8.00 (ddd, $J = 1, 2$ and 8.4 Hz, 1 H, aromatic H), 7.79 (ddd, $J = 8.4$ and 2 Hz, 1 H, aromatic H), 7.56 (ddd, $J = 8.4$ and 2 Hz, 1 H, aromatic H), 4.69 (pentet, $J = 3.3$ and 9.6 Hz, four PEG methylene protons a to the carbonate oxygen), 4.58 (pentet, $J = 3.3$ and 9.6 Hz, four PEG methylene protons a to the carbonate oxygen - possible conformational isomer), 4.28 (very small pentet due to high molecular weight PEG carbonate), 3.92 (pentet, $J = 3.6$ and 9.3 Hz, four PEG methylene protons b to the carbonate oxygen), 3.88 (m, ^{13}C isotope side band), 3.64 (large singlet, PEG backbone), 3.40 (m, ^{13}C isotope side

band); ^{13}C NMR (CDCl_3) δ 146.90 (carbonate carbonyl); 113.03, 132.44, 132.37, 125.98, 115.35, 114.90 (aromatic carbons); 70.47, 70.24 (PEG backbone); 68.12, 67.65.

Example 2: Procedures for the Synthesis of
5 Poly(oxyethylene) bis-(N-hydroxy-2-pyrrolidinonyl) Carbonate 2

The title compound was prepared by following the general procedure described in Example 1, using N-hydroxy-2-pyrrolidinone as the N-hydroxy compound in place of N-10 hydroxy benzotriazole monohydrate. Yield was 93%.

Spectral data: IR (KBr) 2880, 1790, 1732, 1465, 1358, 1278, 1113, 946, 842 cm^{-1} ; ^1H NMR (CDCl_3) δ 4.40 (pentet, J = 2.7 and 9.6 Hz, four PEG methylene protons a to the carbonate oxygen), 3.88 (m, ^{13}C isotope side band), 3.76 (pentet, J = 3.0 and 9.6 Hz, four PEG methylene protons b to the carbonate oxygen), 3.64 (large singlet, PEG backbone and 2 H superimposed, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{CO}$), 3.40 (m, ^{13}C isotope side band), 2.40 (t, J = 7.2 and 8.4 Hz, 2 H, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{CO}$), 2.10-2.22 (m, 2 H, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{CO}$); ^{13}C NMR (CDCl₃) δ 170.41 (NCH₂CH₂CH₂CO), 152.76 (carbonate carbonyl), 70.14 (PEG backbone), 68.56, 68.07, 46.39 (NCH₂CH₂CH₂CO), 26.43 (NCH₂CH₂CH₂CO), 14.95 (NCH₂CH₂CH₂CO).

Example 3: Procedure for the synthesis of
25 poly(oxyethylene) bis-(2-hydroxypyrimidyl) carbonate 3

The chloroformate of PEG 3350 4 was synthesized using the general methods described in Example 1 for the synthesis of carbonate 1, using 2-hydroxypyrimidine hydrochloride as the N-hydroxy compound in place of N-30 Hydroxy benzotriazolemonohydrate as follows.

The crude PEG chloroformate was dissolved in 2.0 L of chloroform and placed under a nitrogen environment. To this solution was added solid 2-hydroxypyrimidine hydrochloride (170 g, 1.257 mol). Tributylamine (300 mL, 35 1.247 mol) was added dropwise. The reaction mixture was brought to reflux and monitored for reaction completion

using proton NMR in CDCl_3 . Monitoring was crucial to ensure that the N-migration product 6 is minimized. At the time that proton NMR revealed all chloroformate had been consumed, the reaction mixture was cooled in a ice bath to precipitate out the excess 2-hydroxypyrimidine hydrochloride. The cold reaction mixture was then filtered through a coarse porosity sintered glass funnel into three 5 liter suction flasks containing ice cold diethylether (3 liters each) which induced precipitation of the carbonate product. The solid was collected by filtration. At this point the solid was contaminated with tributylamine and its hydrochloride salt. These contaminates were removed from the solid by packing the solid in a medium pressure liquid chromatography column and pumping ice cold diethylether through the column until all signs of tributylamine and its hydrochloride salt were absent as monitored by proton NMR. The diethylether used can be recycled into the extraction process by simple rotary evaporation. Once all tributylamine and its hydrochloride salt were removed, the solid was dried in vacuo affording a light yellow solid.

Spectral data: IR (TF, NaCl) 2885, 1775, 1735, 1630, 1465 cm^{-1} ; ^1H NMR (CDCl_3) δ 8.54 (d, $J = 4.8$ Hz, 2 H, pyrimidyl), 7.16 (t, $J = 4.8$ Hz, 1 H, pyrimidyl), 4.10-4.20 (m, four PEG methylene protons α to the carbonate oxygen), 3.68 (m, ^{13}C isotope side band), 3.57 (large singlet, PEG backbone), 3.20 (m, ^{13}C isotope side band); ^{13}C NMR (CDCl_3) d 159.8, 159.6, 151.29 (carbonate carbonyl), 119.28, 69.93 (PEG backbone), 67.79, 66.45.

Urethane N-rearrangement product - Polyoxyethylene bis-(1-pyrimidyl-2-one) carbamate 6: ^1H NMR (CDCl_3) δ 9.19 (d, $J = 8.1$ Hz, 1 H, pyrimidyl), 7.62 (d, $J = 14.1$ Hz, 1 H, pyrimidyl), 5.69 (dd, $J = 8.1$ and 14.1 Hz, 1 H, pyrimidyl), 4.10-4.20 (m, four PEG methylene protons α to the carbonate oxygen), 3.68 (m, ^{13}C isotope side band), 3.57 (large singlet, PEG backbone), 3.20 (m, ^{13}C isotope side band).

Example 4: Determination of hydrolysis half-life ($t_{1/2}$) of PEG carbonates

To a flask of 100 mL of pH 8.5 distilled water using the pH stat (1.0 M aqueous sodium hydroxide) was added 5.0 grams of solid PEG carbonate with vigorous stirring. 10 mL samples were removed at times of 5, 15, 30, 60, 90, 120, 180 and 320 minutes. Each sample was immediately extracted with 25 mL of dichloromethane. The organic phase was dried over anhydrous magnesium sulfate, filtered and evaporated. The residue was applied to a wafer of sodium chloride and an FTIR was obtained. The intensity of the carbonate carbonyl peak was measured compared with the PEG backbone peak located at approximately 1462 cm^{-1} . The ratio of peak intensities was plotted versus time. The time at which the carbonate peak intensity had decreased by 50% as compared with the starting carbonate was determined to be the approximate hydrolysis half life at pH 8.5.

Example 5: Coupling of PEG carbonates with D-glucosamine, sodium heparins and human serum albumin (HSA)

A solution of 288 mg (1.33 mmol) of D-glucosamine hydrochloride in 10 mL of distilled deionized water was adjusted to pH 8.5 with the aid of a pH stat (Metrohm Model 702) using 0.1 M aqueous sodium hydroxide. To this solution was added the PEG carbonate (0.133 mmol) as a solid and the pH stat was allowed to bring the pH back to 8.5. The mixture was stirred for 30 minutes, then the product isolated by extraction with dichloromethane or by ultrafiltration (Amicon membrane YM1: mwco 500) and lyophilization. Yields were higher for product purification and isolation by ultrafiltration.

Sodium heparin reaction with PEG carbonates were conducted in a similar fashion except equal weights of PEG carbonate and sodium heparin were used to ensure complete acylation of all available free amino groups. The crude reaction mixture was ultrafiltrated (Amicon YM1 10,000

mwco) with distilled deionized water using 1.5 liters for every 1.0 gram of PEG carbonate used.

Example 6: Coupling of PEG Carbonates to an Amine Grafted Polymeric Surface

5 The PEG carbonates of the present invention may be conveniently coupled to plasma amine grafted tetramethylhydrocyclotetrasiloxane KDF-190 polypropylene fiber (such as that described in the commonly assigned and concurrently filed U.S. Patent Application "Plasma 10 Grafting Methods and Compounds") according to the following method:

The amine-grafted coated fiber is either dip coated or pulled through a solution of the desired PEG carbonate in dichloromethane having a PEG carbonate concentration of 15 about 3 to about 10% (about 4.25% is preferred). For the dip procedure, contact time is about 10 minutes. For the pull procedure, dwell time is about 10 minutes. The fiber is then removed and washed with dichloromethane. For the pull through process (continuous coating process) a 20 modular coating machine may be used.

Example 7: Coupling of PEG Carbonate Coupled Fiber to Heparin

Carbonate coupled fiber prepared according to Example 6 may be conveniently coupled to heparin using methods 25 similar to those described in Example 5.

For example, a PEG carbonate coupled fiber can be passed through a tank of heparin solution, across a drying area and then wound back on a spool.

Example 8: Determination of heparin mediated Antithrombin III inhibition of Bovine Factor X using the chromogenic substrate S-2222.

Determination of heparin activity of the coated fibers was accomplished using a modification of the commercially available Coatest® assay kit sold by Helena Laboratories.

These modifications included sonication during the incubation period and acid-stop methodology using acetic acid. Sonication during the incubation increased reproducibility with fiber surface bound samples yet had 5 no effect on solution soluble samples. Calibration standardization was relative to the commercially available United State Pharmacopeia K2 heparin standard. Absorbance change was monitored at 405 nm. Solution heparin and heparin-PEG sample activity was expressed as IU/mg of 10 solid. Surface heparin activity was expressed as mIU /cm² surface area. Plasma amine grafted tetramethylcyclotetrasiloxane KDF-190 polypropylene fibers coupled to heparin using one of the PEG carbonates described herein were found to have surface heparin activity which ranged from 15 about 7 to about 14 "IU/cm³.

Table 1. Screening of PEG analogs for acylation with amines: yield^(a) and ι bond ^{13}C chemical shift (ppm)^(b)

PEG Analog	D-glucosamine	D-glucosamine 6-sulfate	N-TMS-al
<u>1</u>	55%, 159.4, 159.1	159.4, 159.2 159.1, 156.6, 155.0	74%
<u>5</u>	<u>2</u> 48%, 159.4, 159.1	159.4, 159.1	48%
	<u>3</u> 90%, 158.7, 158.4	159.4, 159.1, 156.8	98%
	<u>4</u> 62%, 159.4, 159.1	n/a ^(c)	n/a
	<u>5</u> 71%, 159.4, 159.1	159.4, 159.1	n/a

10 (a) - Yields vary from 40-60% when product isolated by aqueous workup using dich 100% when isolated by ultrafiltration (500 MW cutt-off) and lyophilization

(b) - Chemical shift of other amine urethane carbonyls: ammonia -160.11 ppm; glyc

(c) - n/a-not attempted

20

Table 2. Approximate half-life (minutes) of PEG carbonates 1, 2, 3 and 5 as determined by FTIR at pH 8.5

	PEG Analog	Approximate $t_{1/2}$ hydrolysis
5	<u>1</u>	15
	<u>2</u>	132
	<u>3</u>	n/a
	<u>5</u>	47

n/a: N-migration product 6 predominates in absence of an amino nucleophile

Table 3. Fluorescamine analysis of various heparins for primary free amines

Heparin sample		Glucosamine equiv. (nmol/µg (std dev))
Diosynth ^(a)		0.055 (± 0.003)
5	Diosynth (dialyzed) ^(b)	0.053 (± 0.006)
	EDC/diamine modified ^(c)	0.062 (± 0.005)
	N-deacetylated/hydrazide modified ^(d)	0.144 (± 0.023)
10	H ⁺ Dowex treated ^(e)	
	2.5 mL/minute	0.064 (± 0.002)
	5.0 mL/minute	0.065 (± 0.003)
15	N-desulfated modified ^(f)	
	15 minutes, 95°C	0.340 (± 0.026)
	1.5 minutes, 95°C	0.047 (± 0.006)
	15 minutes, 20°C	0.043 (± 0.004)
	15 minutes, 0°C	0.042 (± 0.005)

(a) As purchased from the bottle, average of three triplicate runs

20 (b) Dialyzed from Spectra/Por CE dialysis tubing mwco 1000 against distilled deionized water, average of three triplicate runs

(c) 10.0 grams Diosynth heparin, 32.6 mmol EDC, 326 mmol diamine

25 (d) Diosynth heparin, 6 hours at 100°C, hydrazine and 1% w/v hydrazine sulfate

(e) Fractions tested for toluidine blue and immediately neutralized using 0.1 M NaOH

(g) Diosynth heparin, sulfuric acid (0.23 N)

Table 4. ^{13}C NMR chemical shift (ppm) of natural abundance and 95% ^{13}C ϵ Heparin and PEG-HSA Urethane conjugates, GF-HPLC and Heparin mediated of Factor Xa activity

Urethane ^{13}C signals observed for:				
	Compound	Natural abundance	95% ^{13}C enriched	GF-HPLC ^(a) t_r (minutes)
5	Sodium Heparin ^(b)	--	--	12.00
	<u>1</u> -Na ⁺ heparin ^(b)	158.7, 156.6	159.2, 158.7, 158.3, 156.6	11.85
	<u>2</u> -Na ⁺ heparin ^(b)	158.7, 156.6	159.2, 159.1, 158.7, 158.3, 156.6, 155.1	11.92
	<u>3</u> -Na ⁺ heparin ^(b)	159.3, 158.7, 156.3	160.1, 159.3, 159.2, 158.7, 158.6, 156.7, 156.6, 155.2, 155.1, 153.9, 153.3	11.85
	<u>4</u> -Na ⁺ heparin ^(b)	159.1, 158.9, 158.6	159.2, 158.7, 156.6, 156.4, 155.3	11.82
	<u>5</u> -Na ⁺ heparin ^(b)	159.1, 158.7	159.3, 159.0, 158.9, 158.7, 156.5, 154.0	11.91
10	<u>5</u> -HSA	none	159.2 (Broad)	n/a ^(c)
	<u>1</u> -HSA	none	159.2 (Broad)	n/a

(a) - Gel filtration HPLC and heparin activity testing conducted on natural abundance ^{13}C triplicate

15 (b) - Diosynth, ^(c) - n/a - not attempted

Table 5. Antithrombin III mediated Factor Xa inhibition by surface bound heparin^(a)

Coated fiber run number K2 heparin activity-mlU/cm²
(std dev)

	1	7.8 (4.1)
5	2	9.0 (3.5)
	3	14.0 (3.1)
	4	11.8 (4.3)
	5	10.4 (4.8)

10 ^(a) - Control fiber using a tetramethyltetrahydro-cyclosiloxane coated surface typically afforded values in the range of 0.00-0.3 mlU/cm²

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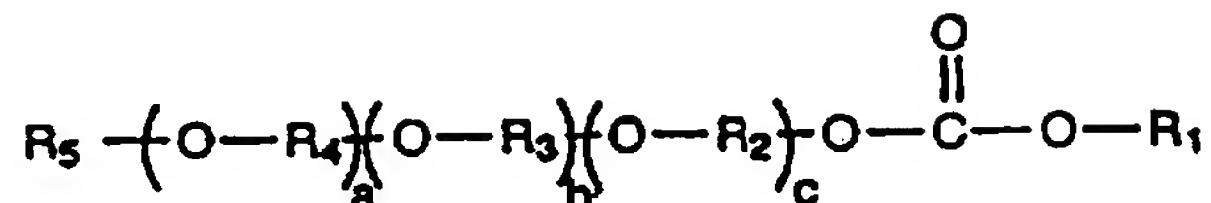
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Claims

1. A compound of the formula:



wherein R_1 is selected from an N-benzotriazole group, an N-2-pyrrolidinone group, or an 2-oxypyrimidine group; R_2 , 5 R_3 and R_4 are independently selected alkylene groups of about 2 to about 3 carbon atoms and may be the same or different; R_5 is selected from hydrogen, methyl, a carbonyloxy-N-benzotriazole group, a carbonyloxy-N-2-pyrrolidinone group, and a carbonyl-10 2-oxypyrimidine group; a is an integer from 1 to 1000 and each of b and c is an integer from 0 to 1000, where $a + b + c$ is 15 an integer from 3 to 1000.

2. A compound of claim 1, where R_2 , R_3 and R_4 are independently $-CH_2CH_2-$ or $-CH_2CH(CH_3)-$ or a combination 15 thereof.

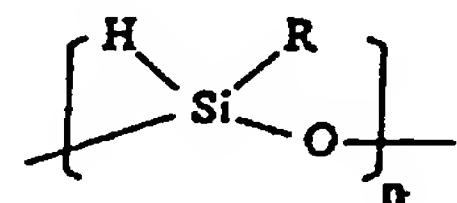
3. A compound of claim 2 wherein R_2 , R_3 and R_4 are $-CH_2CH_2-$.

4. A compound of claim 3 wherein R_5 is selected so as to give a homobifunctional compound.

20 5. A method for the covalent bonding of a bioactive compound selected from an aminoglycan polysaccharide, a peptide and a protein to a polymeric surface using a polyoxyalkylene tether which comprises contacting a substrate having an amine-grafted polymeric surface having 25 free amino groups with a compound of claim 1 to give a modified polymeric surface having activated polyoxyalkylene groups covalently bonded thereto; and

contacting the modified polymeric surface with the bioactive compound.

6. A method according to claim 5 wherein said polymeric surface comprises a membrane formed from plasma 5 polymerization of a hydrocyclosiloxane monomer of the general formula



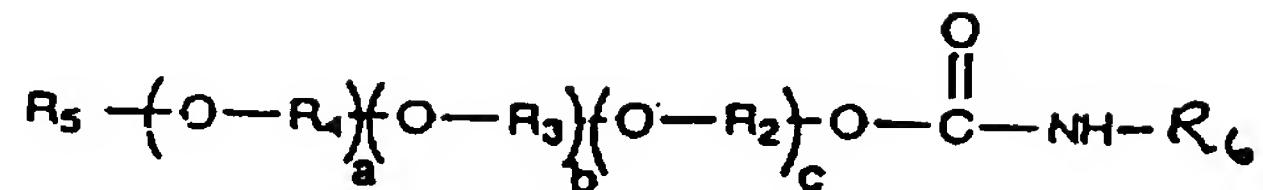
where R is an aliphatic group having 1 to about 5 carbon atoms and n is an integer from 2 to about 10.

7. A method according to claim 6 wherein said 10 hydrocyclosiloxane monomer is selected from the group consisting of 1,3,5,7-tetramethylhydrocyclotetrasiloxane, 1,3,5,7,9-pentamethylhydrocyclopentasiloxane, 1,3,5,7,9,11-hexamethylhydrocyclohexasiloxane, and a mixture of 1,3,5,7,9-pentamethylcyclopentasiloxane and 15 1,3,5,6,9,11-hexamethylcyclohexasiloxane monomers.

8. A method according to claim 7 wherein said free 20 amino groups are grafted on the polymeric surface by reaction with a gas of an N-protected unsaturated or cyclic amine within a plasma chamber under plasma grafting reaction conditions.

9. A method according to claim 8 wherein said N-protected unsaturated or cyclic amine is trimethylsilylallylamine.

10. A compound of the formula



wherein R_2 , R_3 and R_4 are independently selected alkylene groups of 2 to 3 carbon atoms and may be the same or different; R_5 is selected from hydrogen, methyl, a 5 carbonyloxy-N-benzotriazole group, a carbonyloxy-N-2-pyrrolidinone group and a carbonyloxy-2-oxy pyrimidine group; R_6 is an aminoglycon polysaccharide, a polypeptide or a protein; a is an integer from 1 to 1000 and each of b and c is an integer from 0 to 1000 where $a+b+c$ is an 10 integer from 3 to 1000.

11. A compound according to claim 10 wherein R_2 , R_3 and R_4 are independently $-CH_2CH_2-$ or $-CH_2CH(CH_3)-$ or a combination thereof.

12. A compound according to claim 11 wherein R_2 , R_3 15 and R_4 are $-CH_2CH_2-$.

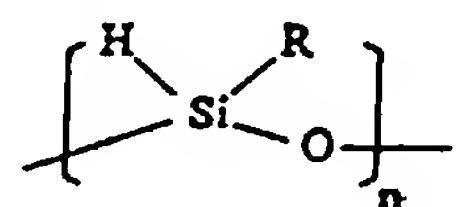
13. A compound according to claim 12 wherein R_6 is heparin.

14. A method of preparing a thromboresistant coating which comprises

20 (a) contacting an object having an amine grafted polymeric surface having primary amine groups with a compound of claim 1; and
 (b) contacting the object with a solution of heparin.

15. A thromboresistant coating made by the method of 25 claim 14.

16. A thromboresistant coating which comprises a membrane formed from the plasma polymerization of hydrocyclosiloxane monomer of the general formula:



5 where R is an aliphatic group having 1 to about 5 carbon atoms and n is an integer from 2 to about 10, covalently linked by a carbamate linkage to one end of a polyoxyalkylene tether wherein the tether is covalently linked at its other end by a carbamate linkage to a heparin molecule.

10 17. A thromboresistant coating according to claim 16 wherein said hydrocyclosiloxane monomer is selected from the group consisting of 1,3,5,7-tetramethylhydrocyclotetrasiloxane, 1,3,5,7,9-pentamethylhydrocyclopentasiloxane, 1,3,5,7,9,11-hexamethyl-15 hydrocyclohexasiloxane, and a mixture of 1,3,5,7,9-pentamethylcyclopentasiloxane and 1,3,5,6,9,11-hexamethylcyclohexasiloxane monomers.

18. A thromboresistant coating according to claim 17 wherein said polyoxyalkylene tether is a 20 polyethyleneglycol.

19. A thromboresistant coating according to claim 18 wherein said polyethylene glycol has a molecular weight of about 500 to 20,000.

FIGURE 1

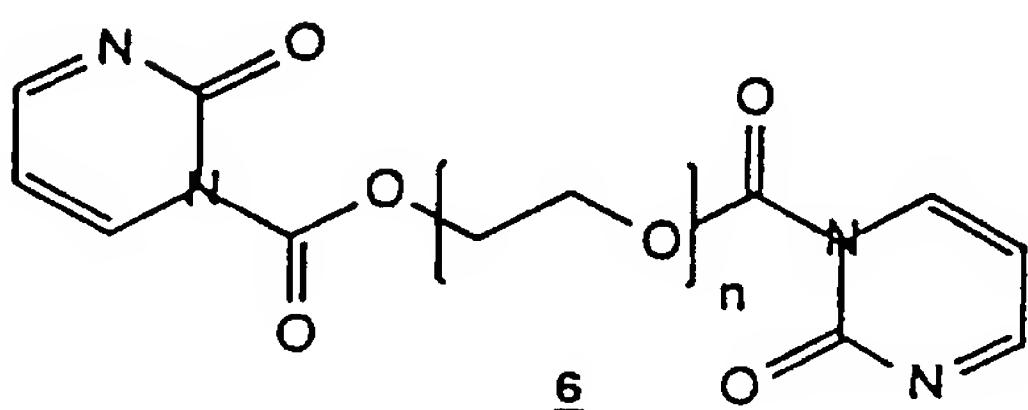
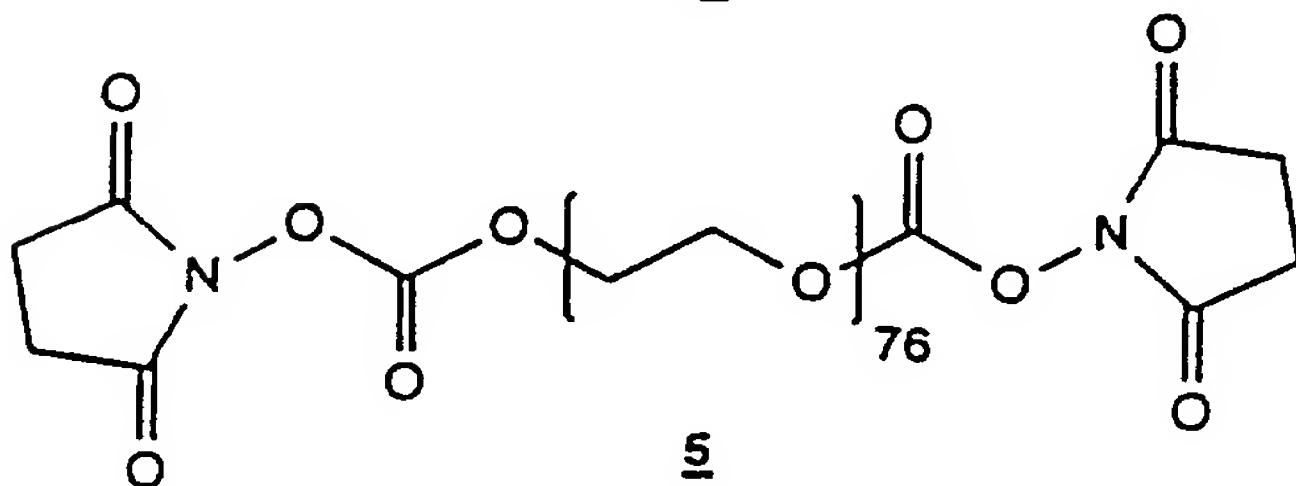
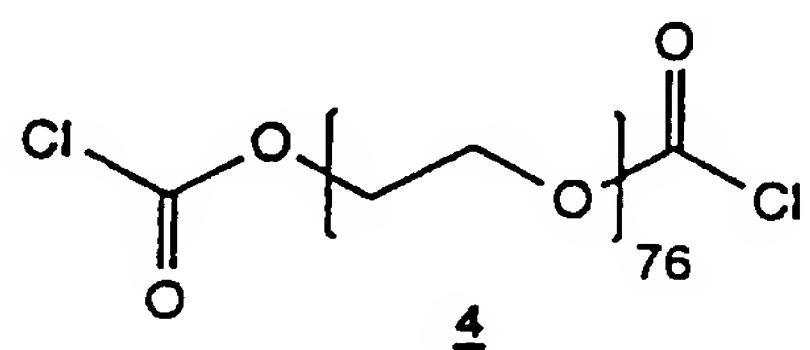
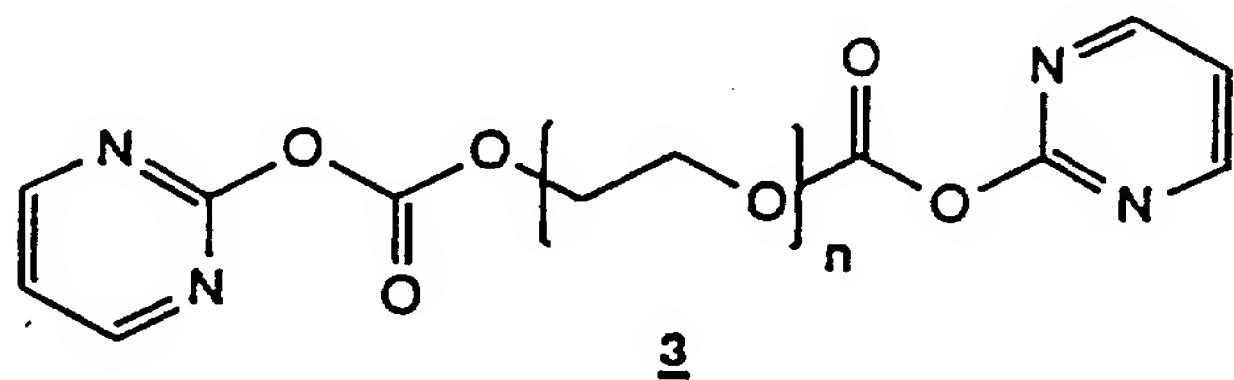
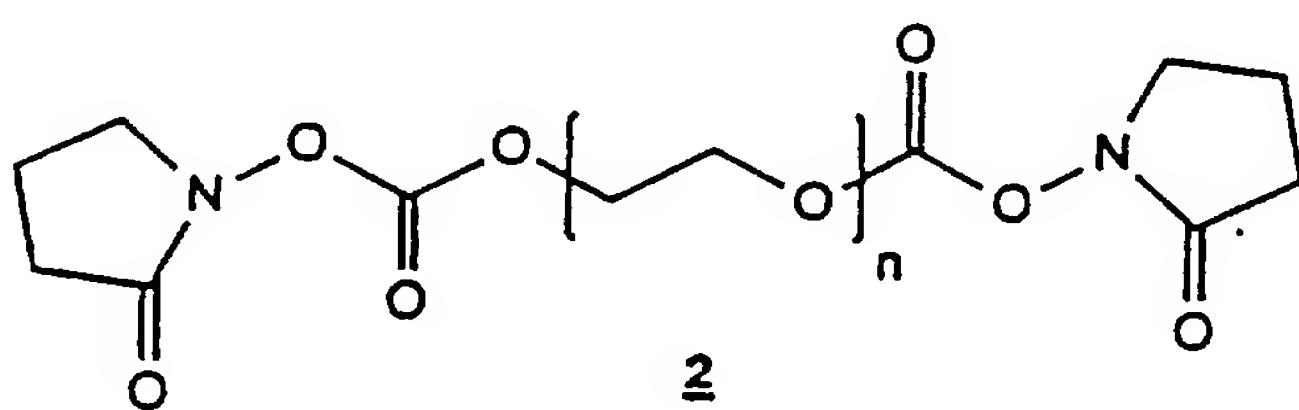
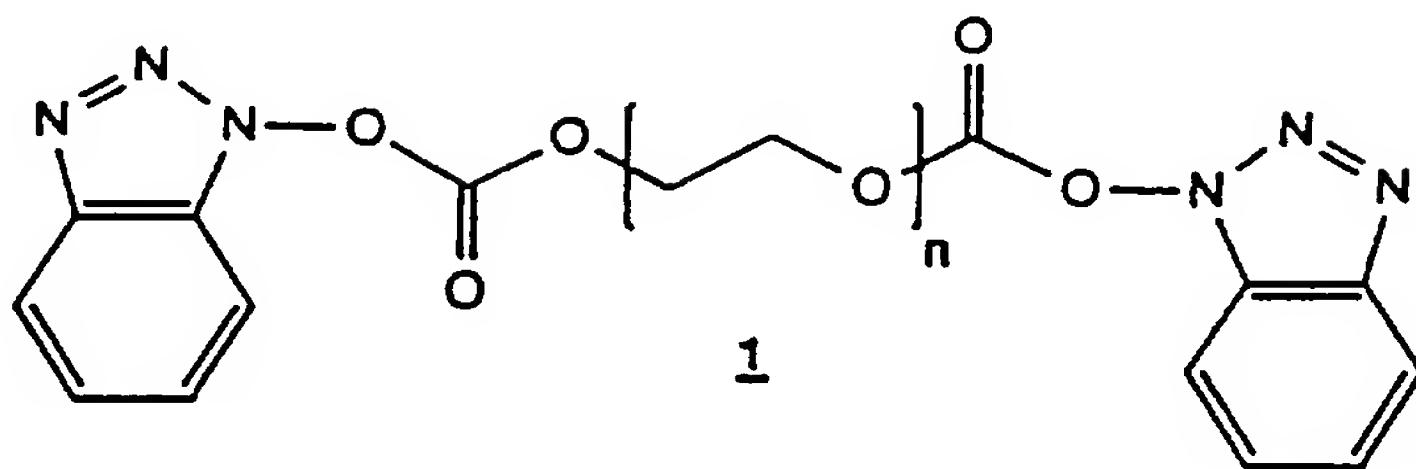
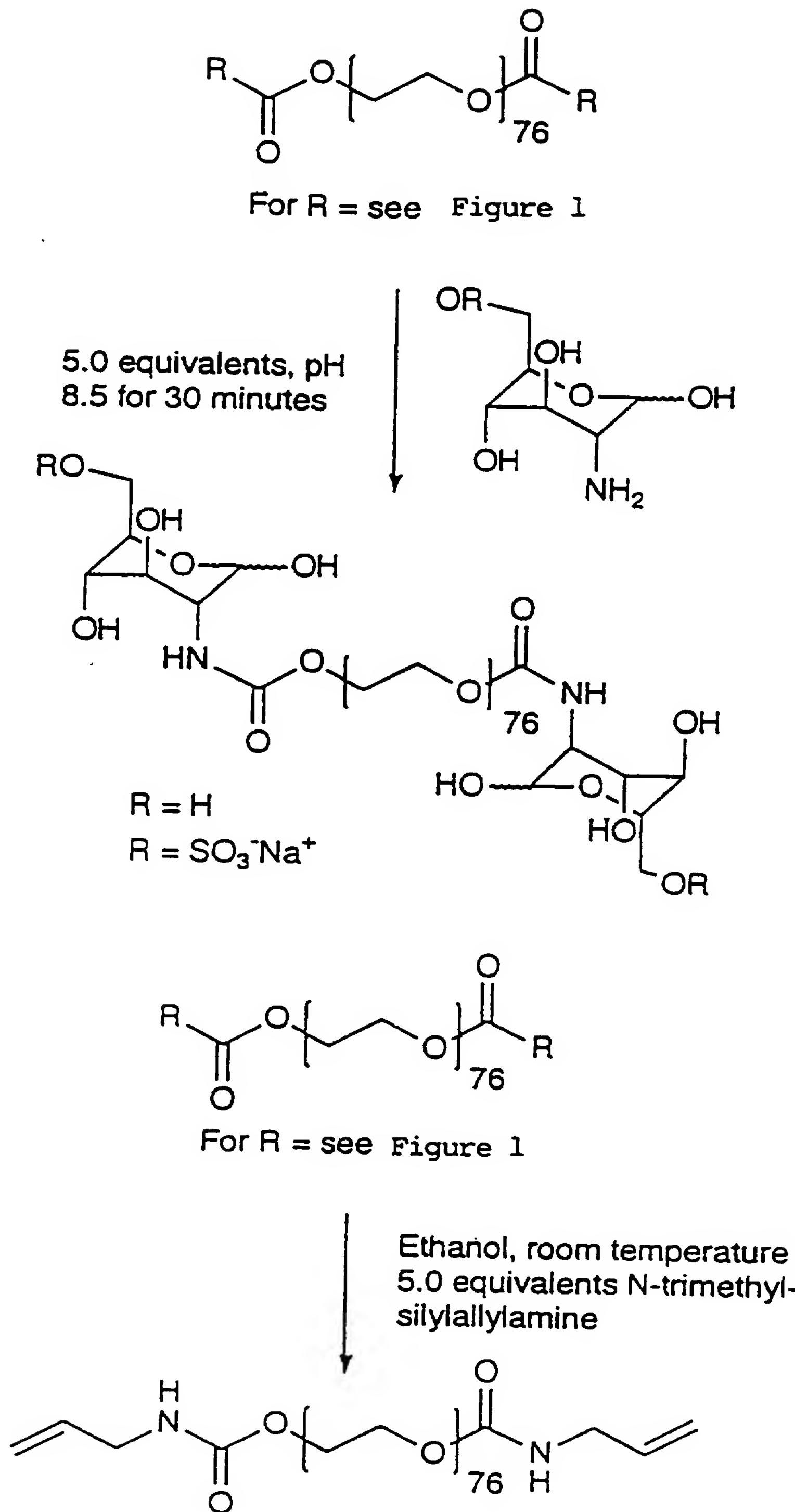


FIGURE 2



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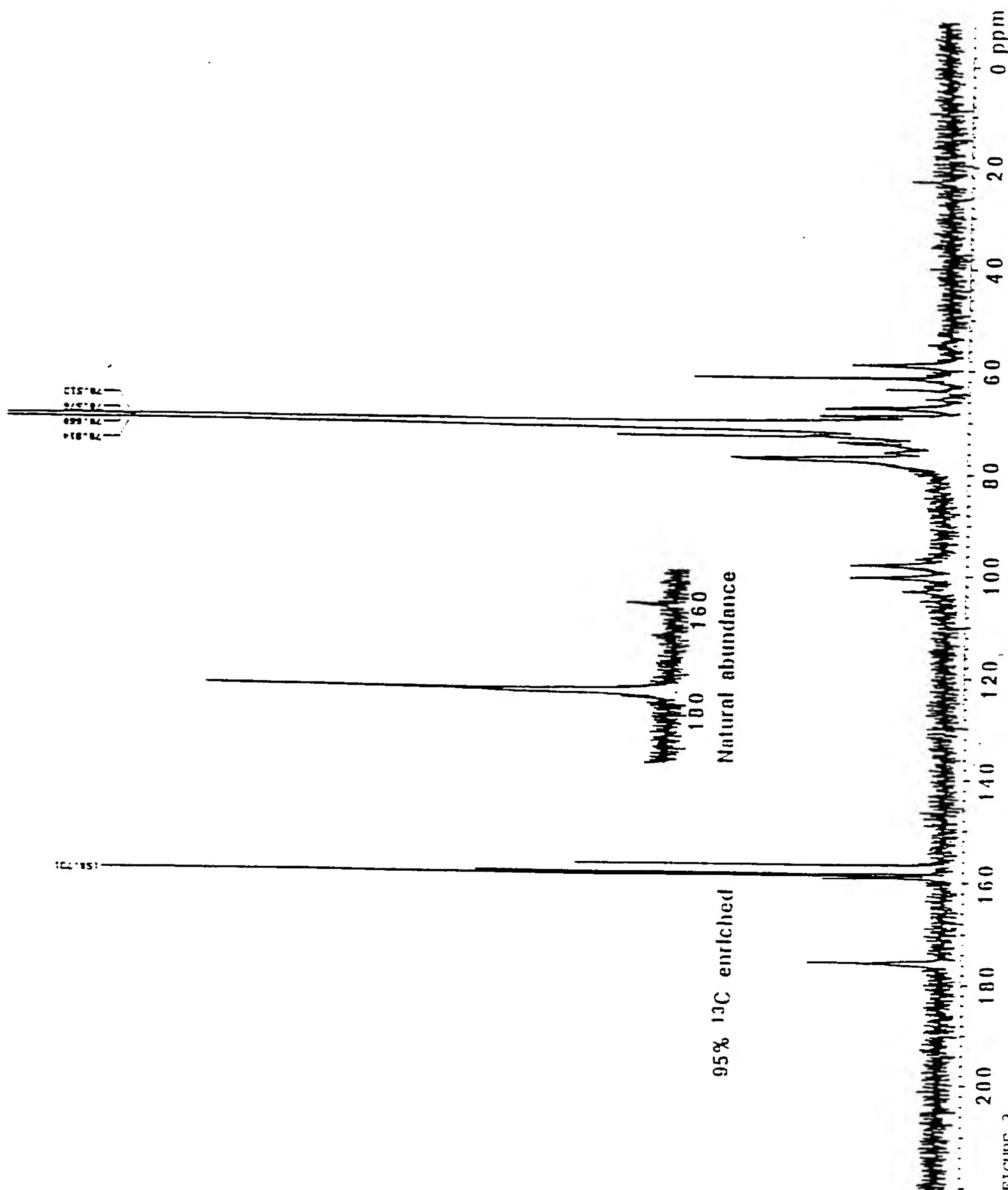


FIGURE 3

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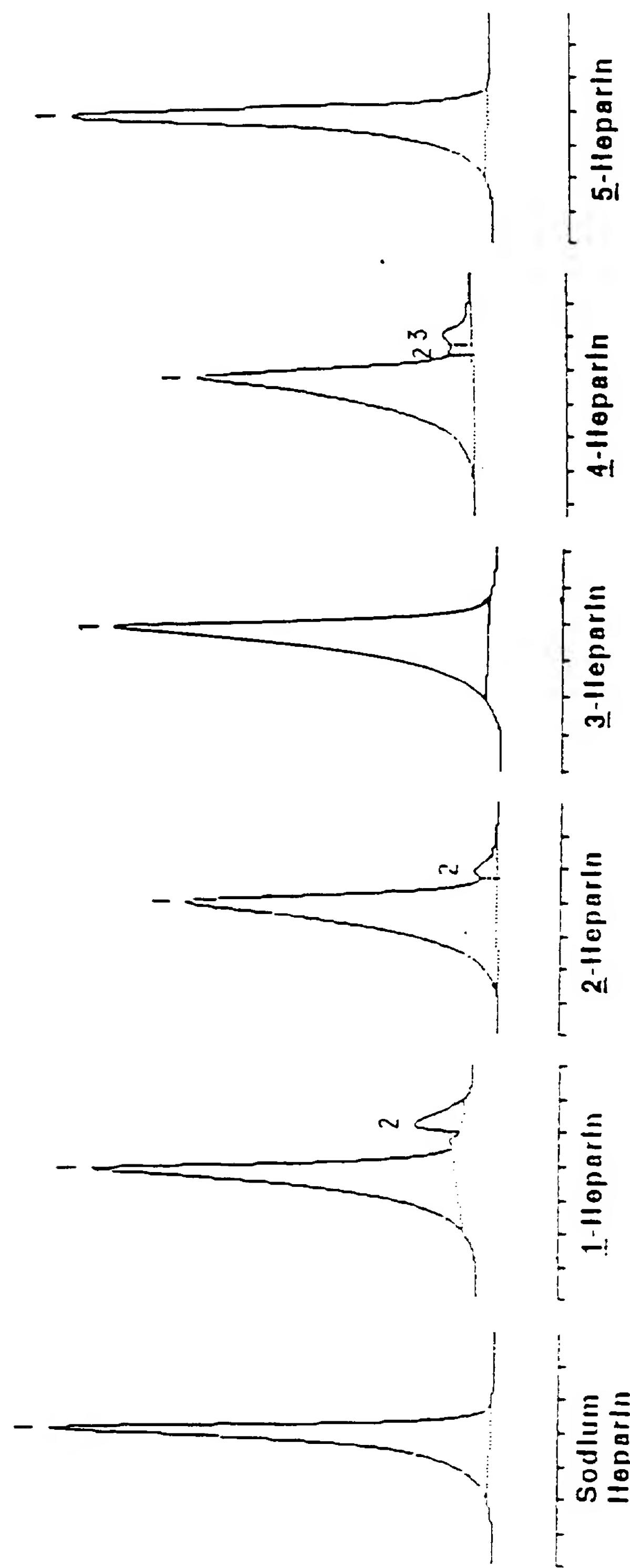


FIGURE 4

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/11255

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07K 1/00, 14/00, 16/00

US CL :530/409, 410, 411; 525/408

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/409, 410, 411; 525/408

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P ----	US, A, 5,349,001 (GREENWALD ET AL) 20 September 1994, column 9, lines 1-10.	10-12 ----
Y,P		13
X	US, A, 5,122,614 (ZALIPSKY) 16 June 1992, column 6, lines 20-50.	10-12

Further documents are listed in the continuation of Box C. See patent family annex.

* Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means		
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Date of the actual completion of the international search

17 OCTOBER 1995

Date of mailing of the international search report

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